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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of
MASAHIRO IMOTO et. al.
Serial No. 10/009,607
Filed: November 11, 2001
For: SUBSTITUTED 1-AZA-2-IMINOHETEROCYCLES AND THEIR USE AS NICOTINIC ACETYLCHOLIN RECEPTORS ACTIVATORS

DECLARATION

I, Yoshihiro Tani, Ph.D., a citizen of Japan residing at 25-9, Tamasecho, Ibaraki-shi, Osaka, 567-0893, Japan, declare as follows.

1. I graduated from Faculty of Pharmaceutical Sciences, Osaka University of Pharmaceutical Sciences in 1984.
2. I graduated from Department of Pharmacology, Graduate School of Pharmaceutical Sciences, Kyushu University in 1986.
3. I entered Suntory Limited as a researcher at the Suntory Institute for Biomedical Research in 1987, and have been assigned as a senior researcher of pharmaceutical research laboratories since 1994.
4. I obtained the Ph.D. in 1991 from Kyushu University.
5. I worked as a researcher at Uppsala University PET center, Sweden from 1991 to 1992.

The following is my opinion regarding the inventions of this application based on my own knowledge applying the technical background.

Neuronal nicotinic receptor agonists have attracted much interest as potential therapeutic agents for the treatment of cognitive impairments associated with Alzheimer's disease, schizophrenia and Parkinson's disease. Clinical studies have revealed that (-)-nicotine is effective to ameliorate memory and attention deficits in Alzheimer's disease patients (Newhouse et al 1986; Sahakian et al 1989; Jones et al 1992). In animals, (-)-nicotine has been reported to show beneficial effects on memory in aged monkeys and to reverse spatial memory deficits in rat with an experimental

lesion of the medial septal nucleus (Levin 1992; Decker et al 1995). In addition, the centrally acting nicotinic receptor channel blocker, mecamylamine, produces significant cognitive impairment that mimics certain aspects of Alzheimer's disease in young and elderly volunteers (Newhouse et al 1994). Postmortem studies of Alzheimer's disease brain tissue demonstrated marked reductions of nicotinic receptors in both neocortex and hippocampus, consistent with the Alzheimer's disease pathology of neuronal degeneration (Araujo et al 1988). These findings point to the functional importance of nicotinic acetylcholine systems in cognitive functions (for recent reviews, see Rezvani and Levin 2001; Newhouse et al 2001).

Regarding the several types of neuronal nicotinic receptor ligands recently discovered, extensive pharmacological and behavioral studies have been carried out on (S)-3-methyl-5-(1-methyl-2-pyrrolidinyl)isoxazole (ABT-418) (Garvey et al 1994), selective agonist at $\alpha4\beta2$ subunits more than $\alpha3$ and $\alpha7$ subunit of neuronal nicotinic receptor (Arneric et al 1994). ABT-418 showed potent cognition-enhancing properties in improving retention of avoidance learning in normal mice and attenuated lesion-induced deficits in a spatial memory in animal model of Alzheimer's disease (Decker et al 1994a; Decker et al 1994b). ABT-418 was the first novel selective nicotinic agonist tested in human patients. In placebo-controlled design study, ABT-418 showed significant dose-related improvement in learning and memory in early to moderate Alzheimer's disease patients (Potter et al 1999). However, ABT-418 is no longer in development by Abbott due to lack of oral bioavailability and the separation between the dosages for the therapeutic effect and the dosages for the potential cardiovascular side effects were too small to be acceptable.

Clinical studies indicate that (-)-nicotine may be beneficial for the treatment of impairment in attention and rapid information processing associated with Alzheimer's disease, and imply that not only the cholinergic system but also monoaminergic systems are possible mechanisms by which (-)-nicotine treatment improves cognitive performance. Among the monoaminergic systems, it has been suggested that noradrenergic effects of stimulants as important therapeutic mechanisms on enhancing capabilities such as attention and working memory.

Therefore, after performing the binding assays at two types of nicotinic receptors and agonist activities at human $\alpha4\beta2$ subunits of nicotinic receptors using Xenopus oocytes, we evaluated the effects of the compounds of the present invention on norepinephrine (NE) turnover in the mouse whole brain as the first *in vivo* assay. It has been reported that brain NE turnover is enhanced by systemic administration of (-)-nicotine in various brain regions and its effect is blocked by the centrally acting nicotinic receptor channel

blocker, mecamylamine (Morgan and Pfeil 1979; Kubo et al 1989). The effects of nicotinic receptor agonists such as (-)-nicotine and ABT-418 were also evaluated as reference compounds.

Materials and methods

Male ddY mice (6 weeks of age, Nihon SLC, Shizuoka, Japan) were used. They were housed in climate-controlled room (room temperature 23±1℃ and humidity 55±5 %) and allowed free access to food and water. Mice were killed by decapitation, after 30 min the compound or drug was administered subcutaneously (s.c.). The mouse whole brain was homogenated in 2.0 ml of 0.1 M perchloric acid containing 0.1 % $Na_2S_2O_5$ and 0.1 % EDTA2Na, followed by the addition of 3,4-dihydrobenzylamine (DHBA) 50 ng as the internal standard. After centrifugation at 1000 g for 20 min, the supernatant was stored at -80 ℃ until assay. The concentrations of NE and 3-methoxy-4-hydroxy-phenylglycol (MHPG), the NE metabolite, were determined by use of a liquid chromatography (LC) system with electrochemical detection. The LC system consisted of a PM-60 pump (BAS) set at 1.2 ml/min, connected to a reverse-phase column (Cosmosil 5C18, 250 mm x 4.6 mm i.d., 5 μm) maintained at 35℃ with a column heater (LC-22A, BAS). NE and MHPG were detected with an electrochemical detector (LC-4B, BAS) set at a potential 750 mV versus the Ag/AgCl reference electrode. The mobile phase was 0.1 M sodium acetate/citric acid buffer, pH 4.80 containing 8 % methanol and 4.6 mM sodium 1-octanesulfonate. The results were statistically analyzed using the Dunnett's two-tailed multiple comparison test. A probability level of $p < 0.05$ was considered significant.

Results and Discussion

The dose-response studies of nicotinic agonists ((-)-nicotine and ABT-418), and 6 compounds (No. 7, 11, 14, 23, 27 and 29) of the present invention on NE turnover in the whole brain of mice were performed (Table 1). (-)-Nicotine increased both MHPG content and MHPG/NE ratio in a dose-dependent manner, and showed significant increases at doses of 1.0 and 5.0 mg/kg. The selective agonist at $\alpha 4\beta 2$ subunits, ABT-418 showed enhancement of both MHPG content and MHPG/NE ratio in a dose-dependent manner, but significant increase was observed at the highest dose of 5.0 mg/kg. The compounds of the present invention also showed significant effects on both MHPG content and MHPG/NE ratio. The compound No.7 and 23 induced significant

increase in NE turnover in a dose-dependent manner, and minimum effective dose was 5.0 mg/kg. The compound No.11, 14, 27 and 29 also exhibited significantly increases of MHPG/NE ratio at 10.0 mg/kg.

Table 1 Effects of (-)-nicotine, ABT-418 and the compounds of the present invention on NE turnover in the mouse whole brain.

Compound	Dose	MHPG	NE	MHPG/NE
	(mg/kg s.c.)	(% of saline group)		
No.7	1.0	105.1±5.1	103.7±1.3	100.8±4.9
	5.0	121.0±9.3	95.8±2.8	126.2±9.3 *
	10.0	133.8±6.2 *	100.4±3.1	133.0±4.9 *
No.11	10.0	116.3±6.8	96.9±2.6	120.1±6.0 *
No.14	10.0	113.0±5.2	95.6±3.1	118.7±5.3 *
No.23	1.0	116.3±4.2	106.2±3.2	109.5±3.9
	5.0	126.3±6.7 *	107.3±5.5	118.2±5.6
	25.0	178.1±10.2 *	106.2±2.5	168.6±13.1 *
No.27	10.0	111.9±4.7	94.2±1.9	119.0±5.2 *
No.29	10.0	112.3±6.8	93.1±2.5	120.0±4.0 *
(-)-nicotine	0.04	106.0±3.7	99.4±3.1	107.1±5.4
	0.2	107.0±6.6	101.8±4.2	105.0±5.9
	1.0	143.5±0.4 *	100.2±4.5	142.3±2.2 *
	5.0	226.9±19.9 *	93.1±3.1	240.5±14.1 *
ABT-418	0.2	104.1±2.6	101.0±3.3	103.3±2.3
	1.0	109.8±4.7	95.9±2.0	114.3±3.7
	5.0	160.1±5.7 *	104.0±2.8	154.6±7.0 *

Animals were killed 30 min after the compound or drug administration. Values in the Table are expressed as percent change from control levels 30 min after saline treatment. * $p<0.05$; significantly different from saline group (Dunnett's two-tailed test, mean ± SEM, n=7-8).

Among novel nicotinic receptor agonists, ABT-418 showed significant improvement both in experimentally induced animal models (Decker et al 1994b) and in early to moderate Alzheimer's disease patients (Potter et al 1999). ABT-418 was a selective agonist at $\alpha 4\beta 2$ subunits of nicotinic receptor, since ABT-418 had high affinity for [^{3}H]cytisine binding (Ki = 3 nM) but was inactive in 37 other receptor,

neurotransmitter-uptake, enzyme, transduction system binding assays, and ABT-418 was equipotent to (-)-nicotine in stimulating [$^{86}Rb^{+}$] efflux from mouse thalamus that was thought to reflect the activation of $\alpha4\beta2$ subunits of nicotinic receptor (Arneric et al 1994). We also confirmed such abilities of ABT-418 using receptor binding assays and Xenopus oocytes expressing $\alpha4\beta2$ subunits of nicotinic receptor.

Regarding the mechanisms by which (-)-nicotine enhanced brain NE turnover, previous our studies have indicated that (-)-nicotine enhances brain NE turnover may be attributed to activation of $\alpha4\beta2$ subunits but not $\alpha7$ nicotinic receptors (Tani et al 2002). Because, (-)-nicotine-induced increase in NE turnover was blocked dose-dependently by pretreatment with dihydro-β-erythroidine (DHβE), a competitive nicotinic receptor antagonist that reported to be more sensitive to $\alpha4\beta2$ subunits, but a selective nACh-R antagonist for $\alpha7$ subunit, methyllycaconitine (MLA) did not affect (-)-nicotine-induced increase in NE turnover. The neuronal nicotinic receptors are thought to be composed of α and β subunit and the most abundant nicotinic receptor in the central nervous system consists of $\alpha4$ and $\beta2$ subunits (Flores et al 1992), while in recombinant expression system $\alpha7$ subunit can form functional homooligomeric receptor. What subunits of nicotinic receptors might mediate cognition-enhancing properties is as yet unclear, but $\alpha4\beta2$ subunits of nicotinic receptor appear to have the greatest relevancy to Alzheimer's disease and other cognitive disorders (Newhouse et al 2001).

The six compounds No. 7, 11, 14, 23, 27 and 29 of the present invention had high affinity for [^{3}H]cytisine binding. Studies with Xenopus oocytes expressing $\alpha4\beta2$ subunits of nicotinic receptor also demonstrated that these compounds had the abilities as agonists for $\alpha4\beta2$ subunits of nicotinic receptor. The present study indicated that a single systemic administration of these compound significantly enhanced brain NE turnover, and the relative potencies for enhancement of brain NE turnover were (-)-nicotine > ABT-418 > compound No.7 and No.23 > compound No.11, 14, 27 and 29. Therefore, these findings suggest that the six compounds No. 7, 11, 14, 23, 27 and 29 of the present invention may exhibit cognition-enhancing properties in both animal model of Alzheimer's disease and patients with Alzheimer's disease.

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**Note: The four references underlined are attached herein.

I, the undersigned petitioner, declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

This 28th day of November, 2003

Yoshihiro Tani

Yoshihiro Tani, Ph.D.

This 28th day of November, 2003

Toshio Tatsuoka

Witnessed by Toshio Tatsuoka, Ph.D.
General Manager